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The Antigonadotropic Activity of a 19-Nor-Progesterone Derivative Is Exerted Both at the Hypothalamic and Pituitary Levels in Women

BEATRICE COUZINET, JACQUES YOUNG, MICHELE KUJAS, GERY MEDURI, SYLVIE BRAILLY, JEAN LOUIS THOMAS, PHILIPPE CHANSON, AND GILBERT SCHAISON

Service d'Endocrinologie et des maladies de la Reproduction et Laboratoire de Biochimie Hormonale, Hopital Bicetre, 94275 Le Kremlin Bicêtre, France; and Laboratoire de Neuropathologie, Hopital Pitié-Salpetrière, 75013 Paris, France

ABSTRACT

We have previously shown in postmenopausal women that a 19-nor-progesterone derivative, nomegestrol acetate (NOMA) had a strong antigonadotropic activity and that this effect was not mediated via the androgen receptor. The aim of the present study was to further assess the action of this progestin on gonadotropin secretion in women. To demonstrate at which level of the hypothalamo-pituitary-ovarian axis the gonadotropin inhibition was exerted, 10 normally cycling (NC) women, 3 women with a gonadotropin-independent ovarian function [McCune-Albright (MCA) syndrome], and 5 women with functional hypothalamic amenorrhea (FHA) participated in the study.

NC women were treated orally with 5 mg NOMA for 21 days, after one control cycle. Plasma estradiol (E_2) and progesterone, LH, and FSH levels were measured during each cycle. A frequent sampling study (every 10 min for 4 h), followed by a classic GnRH test (100 μg, iv), was performed on day 11. Women with MCA were studied before, during NOMA, and after long-acting GnRH agonist administration. In women with FHA, pulsatile GnRH (20 μg sc, every 90 min) was given for two cycles with or without NOMA (5 mg for 21 days).

In all NC women, ovulation was suppressed by NOMA. Mean plasma LH levels, LH pulse frequency, and the LH response to exogenous GnRH were significantly decreased. In MCA, neither NOMA nor GnRH agonist modified multiple ovarian cysts on ultrasound or plasma E_2, levels which remained elevated, ruling out a direct ovarian effect. In FHA, pulsatile GnRH administration recreated a normal ovulatory menstrual cycle. Addition of NOMA prevented the increase of plasma E_2, decreased the amplitude of LH pulses, and prevented ovulation. In view of this unexpected action of NOMA at the pituitary level, seven samples of normal human female pituitaries were tested for the presence of progesterone receptor (PR) using a double labeling immunocytochemical technique. The presence of PR was detected in the seven human pituitary tissues. In addition, PR was found to be expressed only in gonadotroph cells.

In conclusion, NOMA, a 19-nor-P derivative, has a potent antigonadotropic activity exerted at the hypothalamic level, inhibiting ovulation in NC women. In women with FHA, NOMA decreased the gonadotropin stimulation induced by pulsatile GnRH administration. According to the presence of PR in gonadotroph cells of normal human pituitaries, 19-nor-progesterone derivatives may also act on the gonadotropin secretion at the pituitary level. (*J Clin Endocrinol Metab* **84:** 4191–4196, 1999)

IN contrast with classical data (1), the antigonadotropic activity of progestins is not mainly mediated through the androgen receptor. We have previously shown in postmenopausal women that a 19-nor-progesterone derivative, devoid of androgenic, estrogenic, or glucocorticoid effects, displays an antigonadotropic activity as potent as the one observed with nor-testosterone derivatives (2–4). The antigonadotropic activity of Nomegestrol acetate (NOMA; 17α-acetoxy-6α-methyl-19-nor-pregna-4,6-diene-3,20-dione) may be mediated through the progesterone receptor (PR) because it is not suppressed by antiandrogens and is observed in castrated patients with complete androgen insensitivity (4).

As the main inhibitory action of progesterone (P) is exerted at the hypothalamic level (5), the aim of the present study was to further assess at which level of the hypothalamo-pituitary-gonadal axis the gonadotropin inhibition was exerted. For this purpose, three groups of women were studied: 1) normal women with ovulatory cycles (NC) to demonstrate its ability to suppress ovulation; 2) women with gonadotropin-independent ovarian function [Mc-Cune Albright (MCA) syndrome] to demonstrate the absence of effect of NOMA at the ovarian level; 3) women with functional hypothalamic amenorrhea (FHA), to assess after pulsatile GnRH administration, the pituitary or the hypothalamic action of NOMA. The results obtained were unexpected because this progestin displayed a dual impact both at the hypothalamic and pituitary levels. This prompted us to test seven normal human female pituitary samples for the presence of PR in cells of the anterior lobe of the pituitary using an immunocytochemical technique. In front of substantial evidence for PR immunoreactivity, we extended our study and identified with a double immunostaining that PR was selectively expressed in gonadotropin-secreting cells.

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Address correspondence and requests for reprints to: Gilbert Schaison, M.D., Service d'Endocrinologie et des maladies de la Reproduction, Hopital Bicêtre, 94275 Kremlin Bicêtre Cedex, France. E-mail: gilbert.schaison@bct.ap-hop-paris.fr.

Subjects and Methods

Subjects

Ten NC women, 23–35 yr of age, volunteered for the present study. Three women, 18–32 yr of age, who met all the criteria for the MCA

syndrome, gonadotropin-independent secretion of estrogens by the ovary, mutiple ovarian cysts on ultrasound, polyostotic fibrous dysplasia, and café-au-lait pigmented lesions participated in the study.

Five women (22–35 yr of age) with FHA of nutritional origin were also studied. Amenorrhea was defined as the absence of menses for at least 6 months. Magnetic resonance imaging of the hypothalamic-pituitary area was normal. Anorexia nervosa and intensive physical exercise were ruled out. Plasma estradiol (E_2) levels were below 40 pmol/L. None of the patients had withdrawal bleeding in response to P. Plasma FSH and LH were low, and the response to exogenous GnRH (100 μg, iv) showed a peak response of LH release in the range of levels during the early follicular phase, but with a FSH/LH ratio greater than normal.

The protocol was approved by the human investigation committee of the University of Bicetre (Paris, France). The nature of the study was explained, and written informed consent was obtained from all subjects.

Experimental protocols

The 10 NC women were treated orally with 5 mg NOMA for 21 days from day 5 to day 25 of their cycle, after one control cycle. Ultrasonography of the ovaries was performed on day 11 of each cycle. Blood sampling for measurements of plasma E_2 was performed on day 5 and on day 11 of each cycle. On days 18, 20, and 22 of each cycle, plasma E_2 and P were also assayed. On day 11 of each cycle, blood was sampled every 10 min for 4 h (from 0800–1200 h) to measure mean plasma levels of LH and FSH. GnRH stimulation (100 μg, iv) was carried out at the end of each LH pulse analysis. The responses of LH and FSH were measured at 0, 15, 30, and 60 min.

The three patients with MCA syndrome were studied during treatment with NOMA (5 mg/day) for 21 days and long-acting GnRH agonist the following month. The GnRH agonist (microcapsules containing 3.75 mg D-Trp6 GnRH) delivered a controlled daily dose of 100 μg for 30 days. Ultrasonography of the ovaries was performed on day 21 of each treatment. Plasma levels of E_2, LH, and FSH were measured before and on days 10 and 21 of NOMA treatment and of D-Trp6 administration (long-acting agonists induce a maximal gonadotropin inhibition on day 21) (6).

The five women with hypothalamic amenorrhea were studied without treatment, during a treatment cycle with pulsatile administration of GnRH alone for 1 month and in association with NOMA (5 mg/day) for another month. Plasma levels of E_2, LH, and FSH were measured before and during intermittent GnRH pulses administered sc at a dose of 20 μg/pulse every 90 min for two cycles. A portable autoinfusion pump was used (Zyklomat; Ferring Pharmaceuticals Ltd., Paris, France). The pump was left in place for another cycle, and NOMA was given orally at a dose of 5 mg/day. During both treatment periods (pulsatile GnRH administration alone and in association with NOMA), a monitoring of ovulation was performed using ultrasound to follow the appearance of a dominant follicle. Blood samples were drawn every 3 days to measure rapid plasma E_2 levels and plasma P levels.

Hormone assays

Blood samples were immediately centrifuged, and plasma was separated and frozen at −20 C until assayed.

Plasma LH and FSH concentrations were measured in duplicates with a monoclonal antibody immunoradiometric assay (International cis Reagents, Gif sur Yvette, France), as described previously (7). Two hundred microliters of samples were assayed in duplicates. The first antibody was preincubated with the antigen for 2 h at 4 C, then a second antibody was added and incubated for 48 h at 4 C. The assay standards were calibrated against second international reference of pituitary FSH/LH 78/549. The mean detection limit of the assay was 0.025 IU/L. The intra- and interassay coefficients of variation were, respectively, 10% and 13% for concentrations of 0.10 IU/L, 4% and 6% for 2.5 IU/L, and 2% and 3% for 30 IU/L.

Plasma E_2 and P levels were measured in duplicate using established RIA. The intraassay variations were 7% or less for E_2 and P, and the interassay variations of 13% or less for these hormones (7, 8).

Immunocytochemical studies

Immunocytochemical studies were performed to demonstrate the presence of the PR at the pituitary level. All pituitary samples had been obtained from seven female patients undergoing transsphenoidal surgery for pituitary microadenoma (three PRL-secreting adenomas and four ACTH-secreting adenomas). Tissues had been immediately fixed in buffered formalin (10%), processed, and embedded in paraffin. Pituitary sections were cut at 4 μm and stained for conventional light microscopy. At histology, the presence of normal nontumorous adenohypophyseal tissue in the vicinity of pituitary microadenomas was evidenced. Sections with normal nontumoral pituitary tissue available for immunocytochemistry were carefully selected.

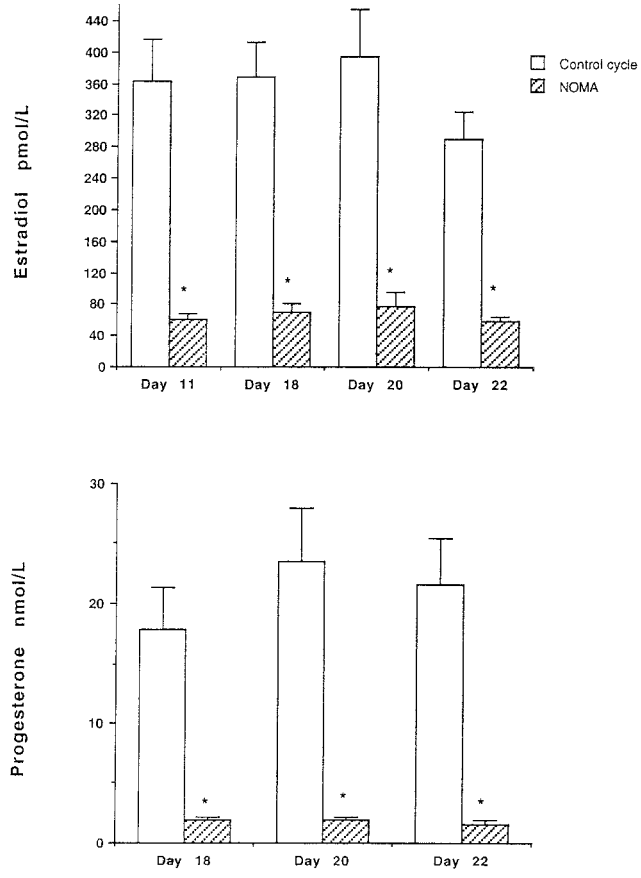

FIG. 1. Mean plasma E_2 and P levels during the control cycle and NOMA administration in 10 NC women. *$P < 0.001$ (*vs.* control cycle).

All experiments were performed simultaneously to minimize risk of technical errors, such as timing, temperature, and dilution. Immunocytochemistry was performed on deparaffinized sections, rinsed in phosphate-buffered saline (pH 7.4), and incubated overnight at 4 C in a humid chamber with a monoclonal anti-PR antibody (DAKO Corp., Trappes, France) at the dilution of 1:100. An immunocytochemical kit was used to detect immunopositivity (Signet, Les Ulis, France). Diaminobenzidine (Eurobio, Les Ulis, France) was used as a chromogen. Control experiments were also performed. Negative controls were incubated with preimmune mouse serum instead of the first antibody. Sections of breast carcinomas of known positivity for PR were included as positive control and simutaneously tested.

PR localization was also investigated in relation to hormone cellular-type using double, nuclear and cytoplasmic, immunostaining. For double immunostaining, the monoclonal anti-PR antibody (1:100 dilution) was used in combination with one of the following antipituitary hormone monoclonal antibodies: anti- hPRL (1:250, Immunotech; Marseille, France); anti-hGH 1:2000, (Signet); anti-ACTH 1–39 1:1000 (DAKO Corp.); anti- α-subunit 1:250 (Serotec, Realef, Varilhes, France); anti-hLHβ 1:250, (Immunotech); anti-hFSHβ 1:300; (Immunotech); anti-hTSHβ 1:300, (Immunotech). The double immunostaining was performed sequentially. After immunostaining for PR as described above, the sections were rinsed in distilled water and immersed in methanol + 3% H_2O_2 for 30 min to eliminate all residual peroxidase activity. The

sections were then extensively washed with phosphate-buffered saline buffer and reincubated with one of the antipituitary hormone antibodies listed above, overnight at 4 C in a humid chamber. The immunoreaction was revealed with a Signet immunostaining kit, and aminoethylcarbazole (Sigma) was used as second chromogen. Single antibody immunolabeling was performed on adjacent sections to compare distribution and intensity of labeling. Negative and positive controls were included as described above. At least five high-power fields were examined for each section. The intensity of immunostaining was quantified as light, medium, and strong.

Statistical analysis

Pulse analysis was done by the program of Thomas *et al.* (9). Pulses can be modeled as innovation outliers, that is as random shocks that affect the underlying process of hormone elimination. This approach leads to a computationally simple iterative procedure that is independent of pulse amplitude distribution and distinguishes between true pulses and gross observation errors.

Gonadotropin-releasing hormone responsiveness was defined as the maximal increase from baseline after the administration of 100 μg GnRH iv. The areas under the curve for each pulsatile profile and gonadotropin responsiveness to GnRH were computed and compared between the spontaneous cycle and NOMA treatment.

All results are given as the mean ± SE. Comparisons of values, between the spontaneous cycle and values during NOMA administration in NC women, between NOMA and D-Trp6 GnRH in MCA subjects, and between pulsatile GnRH alone and in combination with NOMA in FHA subjects were made with the Wilcoxon's rank sum test for matched pairs. Statistical significance was assumed for $P < 0.05$.

Results

NC women

On day 11, NOMA prevented the normal rise of plasma E_2 levels (61 ± 7 pmol/L) compared with the control cycle (363 ± 54 pmol/L; $P < 0.001$) (Fig. 1). On day 11, the mean follicular growth was 15 ± 0.9 mm in the control cycle *vs.* 9 ± 8 mm during NOMA administration ($P < 0.05$). Plasma E_2 and P levels remained very low during NOMA administration, 77 ± 18 pmol/L and 1.9 ± 0.3 nmol/L compared with 394 ± 61 pmol/L and 23.6 ± 5.4 nmol/L, respectively, during the luteal phase of the control cycle ($P < 0.001$) (Fig. 1). Thus, no ovulation occurred during NOMA administration.

During the frequent sampling study, NOMA decreased the mean plasma LH levels from 5.73 ± 0.15 to 3.43 ± 0.11 IU/L ($P < 0.001$), and the LH pulse frequency decreased from 3.2 ± 0.3 to 2.6 ± 0.3 ($P < 0.05$). The area under the curve of the pulsatile LH profile was significantly decreased from 1422 ± 184 to 855 ± 133 IU/min·L^{-1} ($P < 0.05$) (Fig. 2). The LH pulse amplitude was decreased in only 6 of the 10 NC women. However, the decrease of the mean LH amplitude from 2.0 ± 0.3 to 1.8 ± 0.3 IU/L did not reach statistical significance. Mean plasma FSH levels increased from 4.8 ± 0.5 to 6.0 ± 0.3 during NOMA treatment. The area under the curve of FSH pulses increased from 1300 ± 148 to 1841 ± 125 IU/min·L^{-1} ($P < 0.05$) (Fig. 2).

NOMA decreased significantly the LH response to GnRH at 30 and 60 min ($P < 0.01$) with a significant decrease of the area under the curve from 1264 ± 255 to 869 ± 105 IU/min·L^{-1} ($P < 0.05$) (Fig. 3). NOMA increased significantly the FSH response to GnRH with an increase of the area under the curve from 182 ± 20 to 231 ± 20 IU/min·L^{-1} ($P < 0.05$) (Fig. 3).

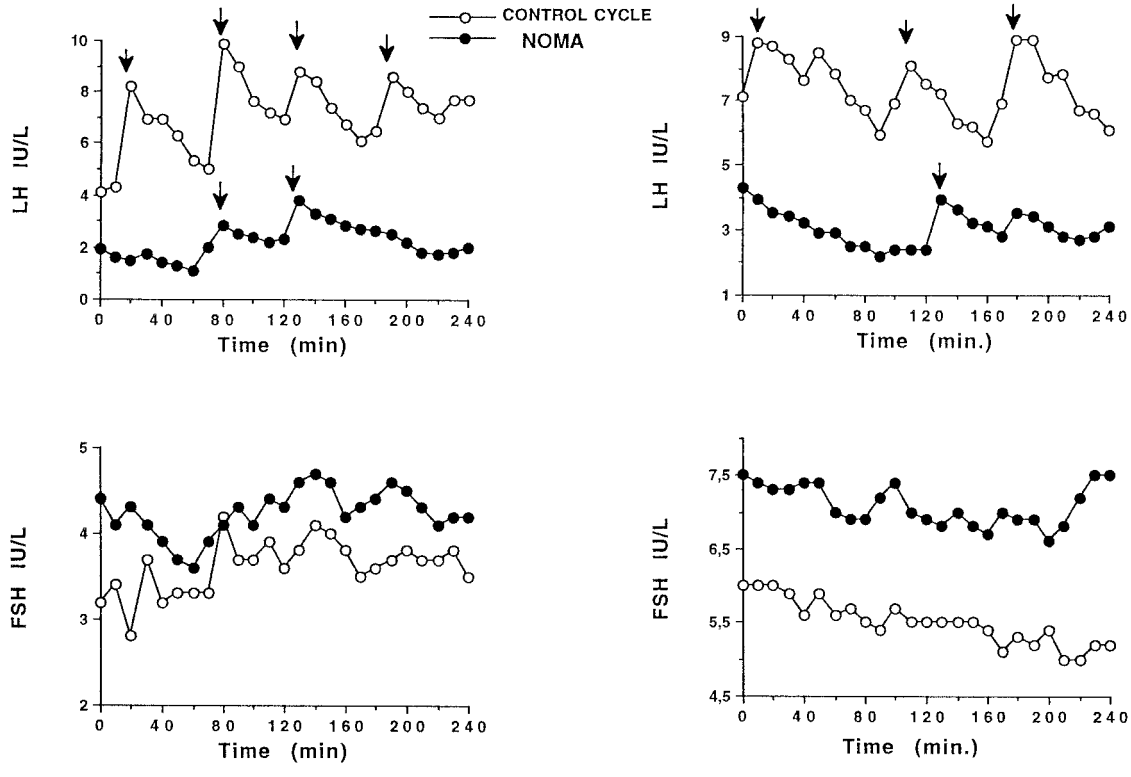

FIG. 2. LH (*top*) and FSH (*bottom*) secretory patterns in two representative normally cycling women on day 11 of the control cycle (○) and on day 11 (●) of NOMA administration. *Arrows* indicate LH pulses.

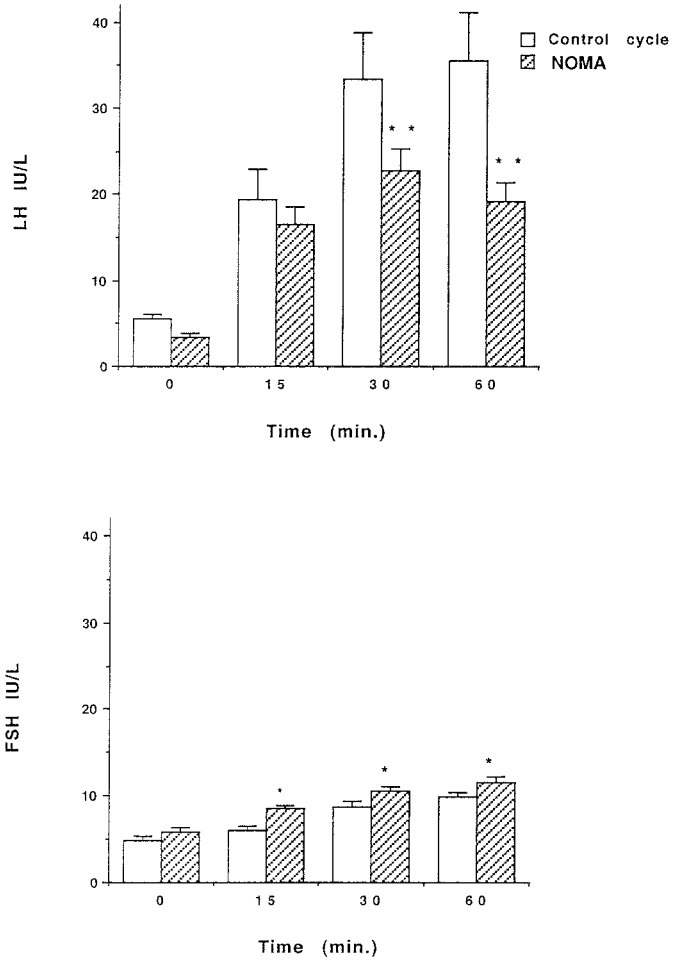

FIG. 3. LH and FSH responses to exogenous GnRH (100 μg, iv) in 10 normally cycling women on day 11 of the control cycle and of NOMA administration. *$P < 0.05$; **$P < 0.001$ (*vs.* control cycle).

Women with gonadotropin-independent ovarian function

In MCA, the ovaries were enlarged (51 ± 3 × 20 ± 2 mm) with bilateral multiple ovarian cysts. Ovarian enlargement (48 ± 2 × 30 ± 3 mm) persisted without reduction of ovarian cysts on ultrasound on day 21 after treatment with either NOMA or GnRH agonist. Plasma E_2 levels were 411 ± 84 pmol/L before treatment and remained elevated during both treatment periods (DTrp6, 392 ± 52 pmol/L; NOMA, 440 ± 92 pmol/L) (Fig. 4). Plasma levels of LH (0.6 ± 0.1 IU/L) and FSH (0.9 ± 0.2 IU/L) were not modified by either treatment.

Women with FHA

In FHA, basal plasma E_2 levels were low (32 ± 11 pmol/L). Pulsatile GnRH administration increased E_2 (to 603 ± 170 pmol/L) and LH levels and induced follicular growth and ovulation in all patients. The addition of NOMA to pulsatile GnRH administration prevented follicular growth and the increase of plasma E_2 levels (99 ± 33 pmol/L) (Fig. 5). No ovulation occurred. During the pulsatile LH studies performed on day 21 of both cycles, a distinct LH pulse was detected after each exogenous GnRH pulse at the frequency (every 90 min), as determined by the pump. During concomitant administration of GnRH pulses and NOMA, the amplitude of LH pulses was almost suppressed, from 6.7 ± 1.8 IU/L to 1.6 ± 0.3 IU/L ($P < 0.005$) (Fig. 5).

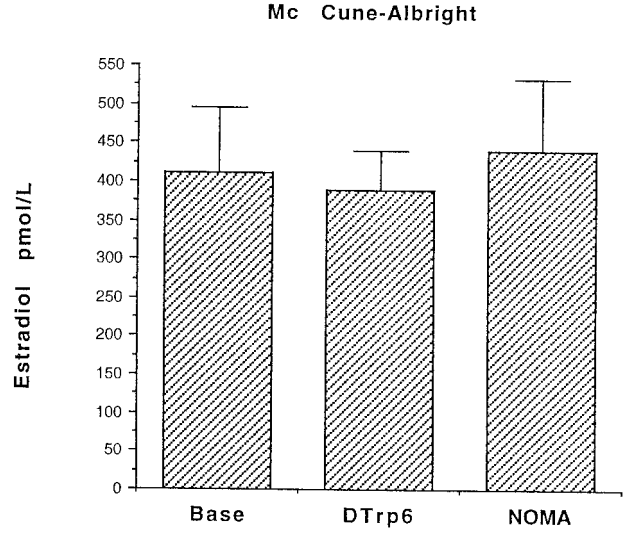

FIG. 4. Mean plasma E_2 levels in women with MCA syndrome before and during NOMA and long-acting DTrp6 GnRH administration.

Pituitary samples

The seven female pituitary samples showed immunopositivity for PR. Table 1 shows the percentage of immunopositivity (from 5–25%; *i.e.*, the number of immunopositive nuclei related to total nuclear number and the intensity of the staining reaction). Fig. 6 shows the positive cells for PR in normal pituitary cords. As distribution of PR was heterogeneous, it was important to mark down the hypophysial cells carrying PR. The seven immunopositive pituitary samples were used for the double immunostaining experiment. Colocalization was observed (nuclear/cytoplasmic) only in cells stained with anti-α, LHβ and/or FSHβ antibodies. However, only about 50% of the total gonadotroph population expressed nuclear immunopositivity with anti-PR (Fig. 6).

Discussion

We have previously shown in postmenopausal women that the dramatic inhibition of gonadotropin secretion induced by 19-norprogesterone and 19-nortestosterone derivatives is similar (4). The present study demonstrates that the antigonadotropic effect of NOMA is also observed in NC women. The inhibition of the hypothalamo-pituitary-ovarian axis, induced by this nor-progesterone derivative, occurred during the first course of treatment. After 11 days, normal folliculogenesis and ovulation were suppressed. Plasma E_2 levels remained very low throughout NOMA treatment, and P levels did not increase. This result has been reported previously (10). Because this compound is devoid of deleterious metabolic effect, its contraceptive effect needs further studies.

The decrease of mean plama LH levels and of the area under the curve of the pulsatile LH profile demonstrated the potent antigonadotropic activity of this progestin. LH pulse

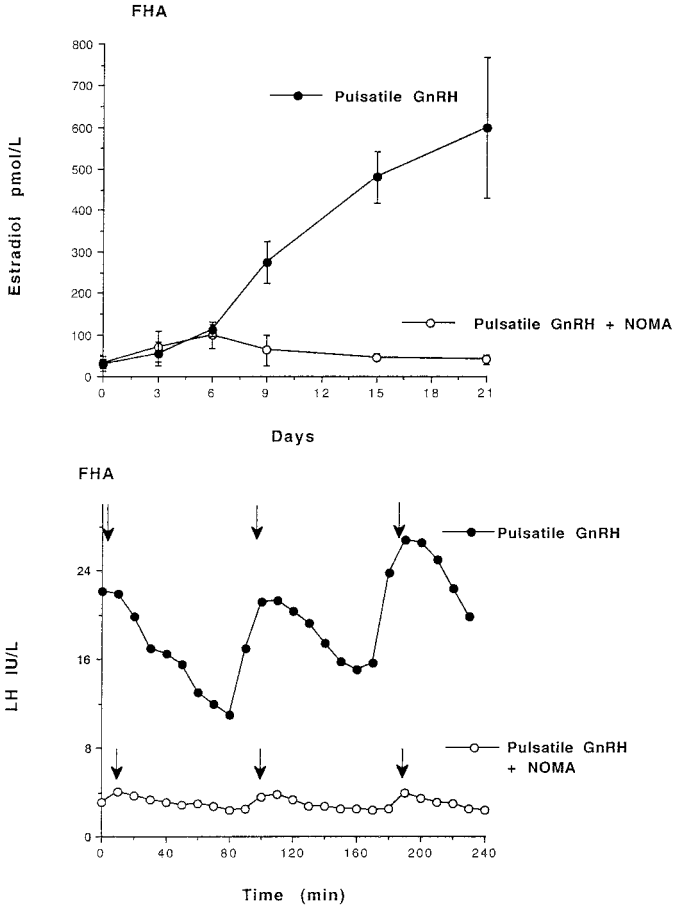

FIG. 5. *Top*, Mean plasma E_2 levels during pulsatile administration of GnRH alone (●) and during concomitant administration of pulsatile GnRH and NOMA (○) in FHA women. *Bottom*, LH secretory patterns in one representative patient with FHA during pulsatile administration of GnRH alone (●) and during concomitant administration of pulsatile GnRH and NOMA (○). *Arrows* indicate LH pulses.

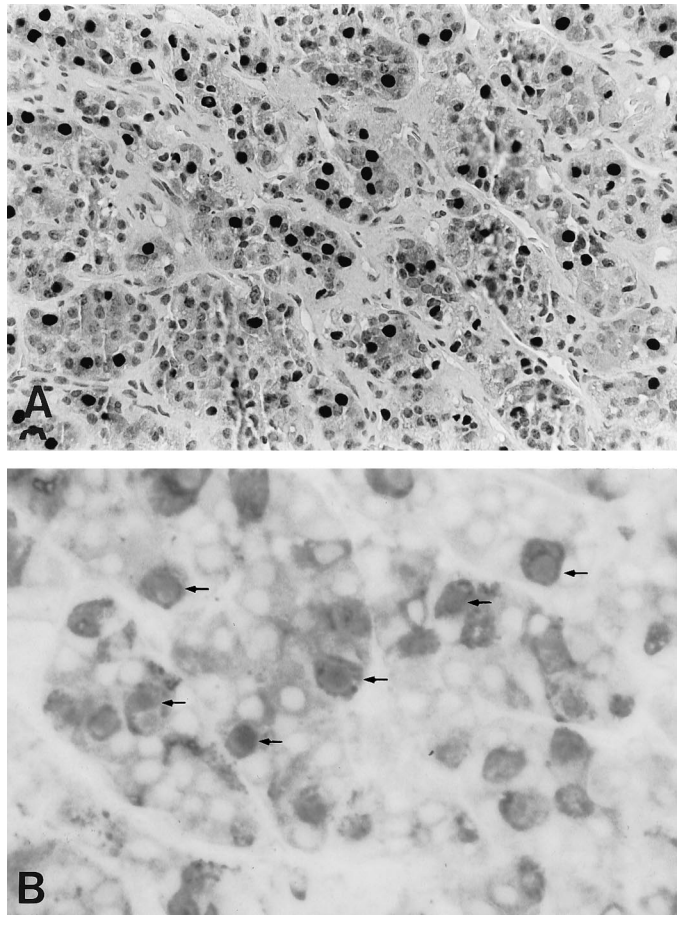

FIG. 6. A, Immunocytochemical staining for PR in normal human pituitary cords; immunoreactive nuclei are dark stained (× 20). B, Double immunostaining with anti-PR and FSHβ antibodies in gonadotroph cells. Only the nuclei of gonadotroph cells are stained with anti-PR antibodies (*arrows*). The nuclei of the other cellular types of the pituitary cords are not stained.

TABLE 1. Immunopositivity for PR and intensity of the staining reaction in seven pituitary samples

No	Percentage of immunopositivity	Intensity[a]
1	25	3
2	20	2 to 3
3	20	3
4	5	2
5	5	2
6	10	2
7	10	2 to 3

[a] Intensity: light (1), medium (2), and strong (3).

frequency and LH pulse amplitude (in 6 of the 10 NC women) were also decreased even with a 10-min sampling for only 4 h. The program of pulse analysis used is independent of pulse amplitude distribution. In addition, in postmenopausal women with higher plasma levels of LH and pulse amplitude, the decrease of LH pulse frequency was obvious with the same protocol of sampling and pulse analysis (4).

The major effect of P is to decrease LH pulse frequency during the luteal phase of the menstrual cycle (10). This action is exerted at the hypothalamic level and may involve endogenous opioids (5). The antigonadotropic activity could also be exerted through the PR. In the female guinea pig, the nuclear presence of PR has been demonstrated in neurons of the hypothalamus and preoptic area (11). A similar hypothalamic action could be considered for NOMA, a 19-nor-progesterone derivative with a selective binding to the PR. We have observed the same decrease of LH pulse frequency with other 19-nor-progesterone or 17-hydroxy-progesterone derivatives (personal unpublished data).

In the present study, mean plasma FSH levels, the FSH/LH ratio, and FSH response to GnRH were increased by NOMA. This result could also be related to the effect of this progestin on the GnRH pulse generator. It is well known that a slowing down of GnRH pulse frequency modifies gonadotropin release, increasing FSH and decreasing LH (5, 12). However, FSH regulation is not exclusively GnRH dependent but relies also on E_2 and intrapituitary peptides (13).

The dramatic decrease in plasma E_2 levels with an increase in plasma FSH levels could suggest a direct ovarian action and suppression of the normal negative feed-back of E_2 on FSH. However, in patients with MCA syndrome receiving

NOMA multiple ovarian cysts remained unchanged on ultrasound, whereas gonadotropin-independent E_2 secretion persisted. This absence of effect of NOMA was similar to the results observed after GnRH agonist administration in these women with autonomous ovarian function. In this model, this negative result ruled out a direct ovarian effect of the progestin. Additional studies are needed to confirm the absence of effect on normal ovaries.

Although the effect of P at the pituitary level seems more controversial compared with its action at the hypothalamic level, we have previously reported in women with FHA that a short exposure to physiological levels of P had a stimulatory effect on LH secretion by acting directly on the pituitary (14). In the present study, the amplitude of LH pulses in normal women during progestin treatment were either unchanged or decreased. It has been previously shown that slow frequencies of GnRH administration produce an increase in pituitary responsiveness (12). Thus, the significant decrease of LH pulse frequency during NOMA administration should have led to an increased pulse amplitude. To further study the hypothalamic and/or pituitary impact of progestins, the clinical model of FHA was useful. In these women, pulsatile GnRH replacement recreated normal menstrual cycle with normalization of LH, FSH, E_2, P, and ovulation. In contrast, during concomitant NOMA and pulsatile GnRH administration, the gonadotropin response to exogenous GnRH was blunted, the amplitude of LH pulses was decreased and plasma E_2 levels remained very low. Unexpectingly, these data showed that the antigonadotropic activity of this 19-nor-P derivative was also exerted at the pituitary level.

The E_2 receptor has been extensively studied in experimental animals and in human pituitaries (15, 16). In contrast, the presence of PR in normal human pituitary has been almost unexplored. In the chicken pituitaries, double immunostaining indicated that PR was present in LH immunoreactive cells and very few PRL cells (17). In the monkey, Bethea *et al.* (18, 19) have demonstrated the presence of PR both at the hypothalamic and pituitary levels. PR was localized only in LH immunoreactive cells (20). In the present study, the presence of immunopositive nuclei for PR was detected in the seven human pituitaries. A second important finding of this study was that, using double immunostaining, PR was found to be expressed only in gonadotroph cells. To our knowledge, this is the first study showing, in the human pituitary gland, a selective expression of PR in gonadotropin-secreting cells. This implies that in addition to the well known effects of P on the inhibitory regulation of GnRH, the steroid is likely to participate in the control of gonadotropin release also at the pituitary level.

In conclusion, 19-nor-progesterone derivatives have a potent antigonadotropic activity exerted at the hypothalamic level, inhibiting ovulation in NC women. A direct ovarian action is unlikely because no effect of this compound was observed in women with autonomous ovarian function. Finally, as plasma E_2 and LH levels did not increase and ovulation did not occur during concomitant NOMA and pulsatile GnRH administration in FHA, a pituitary impact of this progestin may also be proposed. These data are relevant in view of the presence of progesterone receptors detected for the first time in the gonadotrophs of normal human pituitaries.

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